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(54) Title: A NON-MITOGENIC COMPETITIVE HGF ANTAGONIST

(57) Abstract

The present invention relates to a novel truncated form of hepatocyte growth factor (HGF) which specifically antagonizes the activity of HGF. In particular, the present invention relates to the purification, molecular cloning and recombinant expression of the truncated HGF variant. The present invention further relates to the utilization of the small HGF variants in the diagnosis and treatment of diseases in which cell proliferation is either excessive as in the case of malignancy or impaired due in part to aberrant expression of the various forms of HGF.

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A NON-MITOGENIC COMPETITIVE HGF ANTAGONIST

Background of the Invention

This is a continuation-in-part of the application serial number 07/582,063 filed September 14, 1990, the entire contents thereof being hereby incorporated by reference.

Field of the Invention

The present invention relates to a truncated form of hepatocyte growth factor (HGF), encoded by an alternative HGF mRNA transcript, which specifically antagonizes the mitogenic activity of HGF. In particular, the present invention relates to a small HGF variant which functions as a competitive antagonist at the level of HGF binding to its cell surface receptor.

The present invention further relates to methods of diagnosing and treating conditions in which cell proliferation is either excessive, as in the case of malignancy, or impaired, in part due to aberrant expression of the various forms of HGF.

Background Information

Hepatocyte growth factor has hormone-like activity and is released in response to partial hepatectomy and liver injury and is presumed to be an important mediator of liver regeneration (Nakamura et al., *Proc. Natl. Acad. Sci. U.S.A.* 84:6489-6493 (1986); Gohda et al., *J. Clin. Invest.* 81:414-419 (1988); R. Zarnegar and G. Michalopoulous *Cancer Research* 49:3314-3320 (1989)). Its ubiquitous expression by stromal fibroblasts and demonstrated ability to stimulate DNA synthesis in melanocytes and endothelial cells

as well as epithelial cells suggest that this factor plays a role in paracrine regulation of cell growth as well (Rubin et al. *Proc. Natl. Acad. Sci. USA* 88:415 (1991)). Recent reports of the purification of scatter factor, which shows high amino acid sequence identity to HGF over restricted regions, suggests that HGF may also be involved in modulating cell-cell interactions and migration (E. Gherardi and M. Stoker *Nature* 346:228 (1990); Weidner et al. *J. Cell Biology* 111:2097-2108 (1990)).

Structurally, HGF resembles plasminogen in that it possesses characteristic kringle domains (Patthy et al. *FEBS Lett* 171:131-136 (1984)) and a serine protease-like domain (Miyazawa et al. *Biochem. Biophys. Res. Commun.* 163:967-973 (1989); Nakamura et al. *Nature* 342:440-443 (1989)). Like plasminogen, HGF can be processed by proteolytic cleavage, generating a heterodimeric molecule comprised of a heavy- and light-chain covalently linked by disulfide bonds (Nakamura et al., *Proc. Natl. Acad. Sci. U.S.A.* 83:6489-6493 (1986); Gohda et al. *J. Clin. Invest.* 81:414-419 (1988); Zarnegar et al. *Cancer Research* 49:3314-3320 (1989)). The possibility that its actions might be mediated by a receptor tyrosine kinase was suggested by its rapid stimulation of tyrosine phosphorylation of cellular proteins in target cells (Rubin et al., *Proc. Natl. Acad. Sci. USA* 88:415 (1991)). Recent studies have directly identified the HGF receptor as the *c-met* protooncogene product (Bottaro et al., *Science* 251:802 (1991)), whose structure resembles that of a membrane-spanning tyrosine kinase (Park et al. *Proc.*

Natl. Acad. Sci. U.S.A. 84:6379-6383 (1987); Chan et al. *Oncogene* 2:593-599 (1988)).

There is accumulating evidence that the positive effects of growth factors on cell proliferation can be counteracted at a variety of levels both intracellularly (Moses et al. *Cell* 63:245-247 (1990) and at the cell surface (Hannum et al., *Science* 343:336-340 (1990), Eisenberg, et al., *Nature* 343:341-346 (1990); Carter et al., *Nature* 344:633-637 (1990)). Thus, the potential exists to find an antagonist to HGF which would negatively regulate the growth factor's proliferative effects. The invention described herein relates to small HGF variants and their corresponding transcripts. Characterization of one of these HGF variants has revealed that it is a competitive antagonist of HGF action and thus establishes a novel regulatory mechanism whereby the same gene encodes both an agonist and antagonist of growth factor action.

Summary of the Invention

It is an object of the present invention to provide a specific inhibitor of hepatocyte growth factor (HGF) action identified as a smaller form of HGF encoded by an alternative HGF transcript which specifies a sequence that includes the N-terminal and first two kringle domains (HGF/NK2). This truncated HGF variant specifically antagonizes the mitogenic activity of HGF by competitively binding to the cell surface receptor for HGF. The variant itself lacks mitogenic activity.

In one embodiment, the present invention relates to the truncated HGF variant, HGF/NK2 which has an apparent molecular weight of 34 kd by SDS-PAGE under reducing conditions and is substantially free of proteins with which the variant is normally associated.

In another embodiment, the present invention relates to a DNA fragment encoding the 34 kilodalton HGF variant protein.

Another embodiment of the present invention relates to another small form of HGF besides the 34 kd variant that is encoded by an alternative HGF transcript which specifies a sequence that includes the N-terminal and only the first kringle domain (HGF/NK1).

In yet another embodiment, the present invention relates to a DNA fragment encoding HGF/NK1 with a predicted size of approximately 20 kilodaltons.

In a further embodiment, the present invention relates to a recombinant DNA molecule

comprising a fragm nt of the above described DNA and a vector. The invention also relates to a host cell stably or transiently transformed with such a recombinant DNA molecule in a manner allowing expression of the small HGF variant protein encoded in the DNA fragment.

In another embodiment, the present invention relates to a method of producing a recombinant HGF truncated variant with HGF inhibitory activity which method comprises culturing host cells expressing HGF variant protein in a manner allowing expression of the protein and isolating the protein from the host cells.

In a further embodiment, the present invention relates to a method of producing HGF truncated variant protein in cultured cells substantially free of other proteins comprising the steps of culturing HGF variant producing cells in culture medium, contacting HGF variant culture medium with heparin affinity resin under conditions such that a complex between the variant and heparin is formed, separating the complex from the bulk of other protein in the medium, dissociating the HGF variant from the heparin affinity resin and finally fractionating the variant over a sizing column in order to separate any remaining contaminants from HGF variant.

The present invention also relates to cDNA clones that encode the truncated HGF variants, HGF/NK2 and HGF/NK1.

In a further embodiment, the present invention relates to therapeutic applications of the HGF inhibitor variant(s) in proliferative disorders

including both cancer and non-malignant conditions in which HGF is excessive. The method comprises specifically blocking the action of HGF by adminstering a therapeutic amount of HGF inhibitor to a clinical sample or by inducing the endogenous expression of increased amounts of the inhibitor.

The present invention also relates to therapeutic methods that decrease the overproduction of inhibitory HGF variant(s) that are inappropriately produced at high levels in a setting of impaired cell renewal. The method comprises specifically blocking the synthesis or action of the inhibitor HGF molecules by either contacting inhibitor HGF transcripts with antisense oligonucleotides or contacting inhibitor HGF protein with antibodies specific for the inhibitor molecules.

In yet another embodiment, the present invention relates to methods of diagnosing pathological conditions in which cell growth is either impaired or excessive comprising the steps of isolating mRNA transcripts from a biological sample, contacting the mRNA transcripts with a DNA fragment encoding the inhibitory HGF variant, and detecting the presence of specific RNA-DNA hybrids to determine the level of inhibitory HGF variant expression in the sample. The method may also be performed by in situ hybridization in which the step of isolating mRNA transcripts from the sample is omitted before hybridization is carried out.

Various other objects and advantages of the present invention will become obvious from the

drawings and the following description of the invention.

Brief Description of the Drawings

Figure 1 shows the detection of p34 in M426 and SK-LMS-1 cells. Equivalent amounts of [^{35}S]-methionine and cysteine labeled conditioned medium from M426 and SK-LMS-1 cells were immunoprecipitated with non-immune (N) and HGF immune-serum (I). Proteins were subjected to 10% SDS-PAGE under reducing conditions. HGFp87 and p34 are indicated by arrows, and molecular weight markers are shown in kD.

Figure 2 depicts the Northern analysis of RNA from M426 and SK-LMS-1 cells. Two μg of poly(A)$^+$ RNA from SK-LMS-1 and M426 cells were electrophoresed on 1% agarose gels, and Northern blots were hybridized with either HGF coding region (H/L), heavy (H), or light (L) chain probes. The sizes in kilobases (kb) of three major HGF-related transcripts are indicated.

Figure 3 shows the cDNA coding sequence and corresponding amino acid sequence of the 34 kd HGF variant, HGF/NK2.

Figure 4 provides further characterization of a HGF/NK2 cDNA. (A) Schematic representation of the domain structures of HGF and HGF/NK2 (open boxes). The 1.2 kb cDNA clone pH45, comprised of a coding (open bar) and untranslated regions (solid lines). Arrows represent the positions and directions of PCR primers utilized. The cDNA and the predicted amino

acid sequences of HGF/NK2 (EXON) at the point of divergence with HGF are shown with the splice site indicated. The corresponding genomic region (INTRON) includes a ~400bp intron with the consensus splicing signals at the exon-intron boundaries underlined. Abbreviations are: S, signal peptide; N, N-terminal domain; K1-K4, kringle 1 to 4; and L, linker region. Primers are:

P1	agtactgtgcaattaaaacatgcg
P2	gtagaaaaatgattgtatggactgcta
P1(B)	atggatccagtactgtgcaattaaaacatgcg
P2(B)	atggatccgtagaaaaatgattgtatggactgcta
P3	aggcactgactccgaacaggattctttcacccaggcatct cctcc
P4	atggatccttatgtctcgcatgttttaatgcaca

(B) Detection of HGF/NK2 transcript by PCR amplification. Samples included positive control plasmid pH45 (lane 1), RNAs from M426 (lane 2), SK-LMS-1 (lane 3), and B5/589 (lane 4); and genomic DNA from M426 cells (lane 5). Primers P1 and P2 were used in the amplification reactions and PCR fragments (220 and 620 bp) generated are indicated. The faint 620 bp band in lane 3 is indicative of unprocessed HGF RNA or genomic DNA in the SK-LMS-1 RNA preparation.

Figure 5 demonstrates the expression of HGF/NK2 cDNA in COS-1 cells. Conditioned medium from COS-1 cells transfected with plasmid pC45as (antisense construct) or pC45s (sense contruct) as well as M426 and SK-LMS-1 cells were immunoprecipitated with non-immune (N) or HGF antiserum (I). Samples were analyzed under both reducing (A) and nonreducing (B)

conditions. Specific HGF/NK2 immunoreactive species are indicated by arrows.

Figure 6 shows purified naturally occurring HGF/NK2. HGF/NK2 was purified from conditioned medium of SK-LMS-1 cells as described in the Examples. Aliquots from selected fractions eluted from a TSK sizing column were analyzed on 10% SDS-PAGE under reducing condtions (R) or 14% SDS-PAGE under non-reducing conditions (NR) and detected by the silver-stain technique. HGF/NK2 was visualized as a single band migrating at 34 and 28 kD, respectively (Arrows). Higher molecular weight artifactual bands were observed under reducing conditions. An identical sample was subjected to 14% SDS-PAGE under non-reducing conditions and immunoblotted with HGF antiserum.

Figure 7 depicts the analysis of HGF/NK2 biological activity. (A) Comparison of DNA synthethis stimulated by HGF (-O-) and HGF/NK2 (-●-). B5/589 cells were exposed to increasing concentrations of either protein and $[^3H]$-thymidine incorporation was measured as described in the experimental procedures.
(B) Effect of HGF/NK2 on HGF (-O-) and EGF (--●--)-induced $[^3H]$-thymidine incorporation by B5/589 cells. Results are expressed as percentage of stimulation in the absence of HGF/NK2. HGF- and EGF-treated cells were tested at growth factor concenterations (0.2nM and 0.3nM, respectively) in the linear range of their dose-response curves.

Typically, the stimulation was 10,000-20,000 cpm with a background of 2000 cpm.
For both (A) and (B), each data point is the mean ± standard deviation of triplicate measurements; when no error bar is shown, the error was less than the symbol size.

Figure 8 shows the cross-linking and competition analysis of HGF/NK2 to the HGF receptor. [^{125}I]-HGF/NK2 was incubated with B5/589 cells in the presence or absence of HGF/NK2, HGF, or EGF at the concentrations indicated for 45 minutes at 22°C. Cultures were then washed with HEPES saline and incubated for 15 minutes with the cross-linking agent, disuccinimidyl suberate. Total cell lysates were resolved by 6.5% SDS-PAGE under reducing conditions and the dried gel was exposed to X-ray film at -70°C for 32 days.

Figure 9 shows the cDNA coding sequence and corresponding amino acid sequence of the HGF varient encoded by the 1.5 and 2.2 kb transcripts, HGF/NK1.

Detailed Description of the Invention

The present invention relates to a truncated form of hepatocyte growth factor (HGF), encoded by alternative HGF transcripts which specify a sequence that includes the N-terminal and first two kringle domains. This protein specifically antagonizes the mitogenic activity of HGF. The present invention also relates to another truncated form of HGF encoded by alternative HGF trancripts which specify the sequence that includes the N-

terminal and only the first kringle domain (HGF/NK1). The invention further relates to diagnostic and therapeutic applications of the small HGF inhibitor.

A principle embodiment of the present invention relates to a truncated variant of HGF that is synthesized in cells that also normally synthesize HGF. One such HGF variant is characterized by a molecular weight of about 34 kd as determined by SDS-PAGE under reducing conditions. The molecule lacks mitogenic activity but specifically inhibits HGF induced mitogenesis by competing with the growth factor for binding to the HGF receptor.

The HGF variant and HGF protein sequences are > 99% identical throughout the entire length of the smaller HGF variant molecule. The truncated HGF and allelic variations thereof represent the product of an alternative transcript derived either from the same genetic locus encoding HGF or from a recently duplicated gene copy. This conclusion is supported by findings that not only the NK2 coding sequence but its upstream 5'-untranslated region are identical to that of the HGF cDNA. Further evidence shows that the K2 (kringle two) sequence is contiguous in human genomic DNA with the exon containing the termination codon and polyadenylation signal for the NK2 transcript (Figure 4(A)).

The HGF variant protein to which the invention relates can be isolated from conditioned medium of a human leiomyosarcoma cell line as well as other cell lines, for example, M426 fibroblast line, substantially free from other proteins.

Following the instructions pr sented herein, an active form of inhibitory HGF variant of the present invention can be obtained by a combination of protein purification steps that include concentrating the conditioned medium, applying the concentrate to heparin supports, for example, heparin-Sepharose resins, and eluting the HGF variant with an increasing salt gradient. Purified HGF variant is realized after the heparin bound eluate is fractionated over a sizing column, for example, TSK-G3000, in order for the HGF variant to be separated from any remaining components in the eluate. Alternatively, the variant can be produced chemically or recombinantly using methods known in the art.

The present invention also relates to the cDNA clones that encode the truncated HGF variants, HGF/NK2 and HGF/NK1. By screening a M426 human lung fibroblast cDNA library with DNA probes specific for either the heavy or light chain region of HGF, four cDNA clones were identified that hybridized to the heavy but not the light chain probe. Two of these four clones, having inserts of 1.2 or 1.6 kb, contain the coding sequence for the inhibitory HGF variant, HGF/NK2; they differed from each other in length of their 3'-untranslated sequence. However, the other two clones contained inserts of 1.5 and 2.2 kb, respectively, and each of which encoded only the N-terminal and first kringle domain; they differed from each other in their 3'-untranslated region. The resultant truncated form of HGF, HGF/NK1, has a predicted molecular weight of approximately 20 kilodaltons and is anticipated to

have specific HGF inhibitory activity like HGF/NK2. The Northern blot analysis of HGF expression in M426 and SK-LMS-1 human cell lines revealed a weak 2.2 kb band as well as a diffuse signed at 1.3-1.5 kb (Figure 2) which probably represent the transcripts corresponding to these low abundance cDNAs.

The present invention further relates to recombinant DNA molecules comprising a vector and DNA fragment which encodes either of the human truncated HGF variants, HGF/NK1 or HGF/NK2. Possible vectors include plasmids, for example, pCDV-1 and other vectors such as pZIPneo, known in the art that either transiently (pCDV-1) or stably (pZIPneo) transform host cells in a manner which allows expression of the HGF variant. Examples of appropriate eukaryotic host cells include, for example, mouse fibroblasts and monkey epithelial cells. The bacculovirus as well as other eukaryotic or prokaryotic expression systems could be adapted for the production of the HGF variant.

The present invention also relates to therapeutic applications of truncated forms of HGF to which the invention relates, such as HGF/NK2, which has been shown to inhibit the mitogenic activity of HGF. Use of a specific inhibitor of HGF action can be beneficial in treating proliferative disorders, including both cancer and non-malignant conditions like benign prostatic hypertrophy, when HGF stimulation is excessive. The inhibitory HGF variant of the invention can be administered by different routes, for example, topical, oral or intravenous, to patients with such proliferative disorders. It is expected that providing

therap utic amounts of inhibitory HGF variant will return cell proliferation to normal levels. Alternatively, situations in which the production of the inhibitory HGF variant(s) is inappropriately high with a resultant impairment in cell proliferation or renewal can be addressed by specifically blocking the synthesis or action of these molecules (i.e., by use of antisense oligonucleotides to the unique 3'-untranslated sequences or antibodies specific for the inhibitory molecules).

The present invention also relates to methods of diagnosing pathological conditions in which cell growth is either impaired or excessive, due at least in part to the level of expression of HGF and its inhibitory variant(s). Fluctuating levels of these transcripts, particularly of the 1.3 kb transcript relative to the transcript encoding mitogenically active HGF, have been observed in different cell lines in a manner which may correlate with a functional role in regulating proliferation. For instance, the 1.3 kb transcript is expressed at relatively low levels in an embryonic fibroblast line which supports active cell division, but the transcript is present at much higher levels in an adult fibroblast strain which is likely to provide a more attenuated stimulus of cell renewal. As one skilled in the art will appreciate, increased protein production can result from increased levels of corresponding mRNA transcripts. Using DNA fragments derived from the cDNAs of HGF variants and standard methodology known in the art, HGF variant transcripts can be detected as shown in Figure 2.

Detection may be performed with extracted RNA or by _in situ_ hybridization using the DNA fragments or RNA fragments derived therefrom.

In another detection method for diagnosing pathological conditions in which cell growth is either impaired or excessive, a biological sample from a patient is contacted with antibodies specific for HGF and/or specific HGF variants. Using standard methodologies well known in the art, the antibody-protein complex can be detected, for example, by immunoprecipitation and SDS-polyacrylamide gel electrophoresis (Figure 1), immunoblotting (Figure 6), enzyme-linked immunosorbent assay (ELISA) or immunohistochemistry.

Certain aspects of the invention are described in greater detail in the non-limiting Examples that follow.

Examples

The protocols described below are referenced in the following Examples.

Cell culture

Cells including the M426 human embryonic lung fibroblast (S.A. Aaronson and G.J. Todaro *Virology* 36:254-261 (1968), SK-LMS-1 human leiomyosarcoma (J. Fogh and G. Trempe *In: Human Tumor Cells In Vitro*, J. Fogh (ed.), Plenum Press, New York 115-159), and COS-1 monkey kidney epithelial (Gluzman et al. *Cell* 23:175-182 (1981) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) suplemented with 10% fetal calf serum (Bethesda Research Laboratories). B5/589 human mammary epithelial cells (M.R. Stampfer and J.C. Bartley *Proc. Natl. Acad. Sci. U.S.A.* 82:2394-2398 (1985) were grown as described (Rubin et al., *Proc. Natl. Acad. Sci. USA* 86:802 (1989))

Mitogenic assays

DNA synthesis was measured as previously described (Rubin et al., *Proc. Natl. Acad. Sci. USA* 86:802 (1989)). Ninety-six well microtiter plates were precoated with human fibronectin at 1 $\mu g/cm^2$ prior to seeding with B5/589 cells. [^{3}H]-thymidine incorporation was determined during a 6-hr period beginning 16 hr after addition of samples. Trichloroacetic acid-insoluble DNA was collected and counted. HGF used in this study was purified in this laboratory as has been reported (Rubin et al., *Proc. Natl. Acad. Sci. USA* 88:415 (1991)), and human

recombinant EGF was purchased from Upstate Biotechnology Inc.

Immunoprecipitation

Cells in 100mm tissue culture plates were labeled with 0.1mCi/ml of [^{35}S]-methionine and cysteine (spec. act. 1150Ci/ml; Du Pont-New England Nuclear) in 50μg/ml of heparin for 4 hrs as previously described (Rubin et al., *Proc. Natl. Acad. Sci. USA* 88:415 (1991)). Conditioned medium was concentrated 20-fold in Centricon-10 microconcentrator (Amicon) and immunoprecipitated with nonimmune or HGF neutralizing antiserum. Immunoprecipitates were absorbed onto Gamma-bind G agarose (Genex) and washed three times with 10mM Tris-HCl buffer containing 150mM NaCl, 0.05% Tween-20, 0.1% SDS, 1% NP-40, 1mM EDTA, and 10mM KCl. Samples were analyzed under reducing (with 100mM ß-mercaptoethanol) and non-reducing conditions on 10% or 14% SDS-PAGE. Gels were fixed, treated with enlightening solution (New England Nuclear), dried, and exposed to Kodak AR film at -70°C.

Northern analysis

Poly(A)$^+$RNA was isolated by oligo-dT columns as described (Maniatis et al. *Molecular cloning. A Laboratory Manual* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1982)). Following electrophoresis in 1% denaturing formaldehyde agarose gels, samples were transferred onto nitrocellulose filters (Maniatis et al. *Molecular cloning. A laboratory Manual*(Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1982)). Blots were

hybridized to [^{32}P]-labeled randomly-primed DNA probes in 40% formamide, 6x SSC, 5x Denhardt's solution, 50mM sodium phosphate (pH6.8), and 250μg/ml of sonicated salmon sperm DNA at 42°C for 12 hrs. After hybridization, filters were washed twice in 1x SSC, 0.1% SDS at room temperature. The final wash was carried out in 0.1xSSC, 0.1% SDS at 55°C. Filters were dried and exposed to X-ray films for 5-8 days at -70°C. Hybridization probes were generated by PCR and purified on low-melting temperature agarose gels. The nucleotide sequence of each probe was numbered according to the HGF sequence of Miyazawa et al. *Biochem. Biophys. Res. Commun.* 163:967-973 (1989) as follows:

H/L (heavy and light chains): -24 to +2187
H (heavy chain) : +189 to +1143
L (light chain) : +1475 to 2122

<u>cDNA cloning and sequencing</u>

Approximately 1x10^{6} phage plaques from an M426 cDNA library (Finch et al. *Science* 245:752-755 (1989) were plated, and duplicate filters were hybridized separately to radiolabeled probes H and L (see above) under conditions identical to those described for Northern analysis. Restriction mapping of plaque purified positive clones was performed using standard procedures (Maniatis et al. *Molecular cloning. A laboratory Manual* (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1982)). cDNA inserts were excised and subcloned into the M13mp18 vector for sequencing analysis by the dideoxy chain-termination method (Sanger et al., *J. Mol. Biol.* 143:161-178 (1977).

PCR analysis

For PCR of mRNA, 1µg of poly(A)⁺ RNA was first reverse-transcribed by avian myeloblastosis virus(AMV) reverse transcriptase (Bethesda Research Laboratories) using random hexamers (Pharmacia) as primers (Noonan et al. *Nucleic Acids Res.* 16:10366 (1988)). Eight percent (~80ng) of the first-strand cDNA products were used directly in PCR (Saiki et al. *Science* 230:1350-1354 (1985)). For routine PCR, 80ng of cDNA, 0.5µg of cellular DNA, and 10ng of plasmid DNA were subjected to 30 cycles of amplification using primers P1 and P2 (see Figure 4). Cycling conditions were: 1 minute at 94°C, 2 minutes at 60°C, and 3 minutes at 72°C. Aliquots (10%) of each reaction mixture were anlyzed on 3% agarose gel. For PCR cloning of genomic DNA, PCR was carried out with BamHI linker-primers P1B and P2B (Figure 4) and amplified DNA fragments were digested with BamHI. The resultant BamHI fragments were purified on low-melting temperature agarose gel and subcloned into the M13mp18 vector for sequencing analysis.

Transient expression in COS-1 cells

The NK2 coding sequence was generated by PCR using BamHI linker-primers, P3 and P4 (Figure 4) and subcloned into the BamHI site of the vector pCDV-1 (Okayama et al. *Mol. Cell. Biol.* 3:280-289 (1983)) in both orientations. The NK2 insert in a selected construct was sequenced to ensure that the PCR product was correct. Ten µg of each plasmid DNA was transfected by the calcium phosphate precipitation method (Wigler et al. *Cell* 11:223-232 (1977)) into

COS-1 cells (Y. Gluzman *Cell* 23:175-182 (1981)). At 48 hrs, proteins in conditioned medium were processed for labeling, immunoprecipitation and 10% SDS-PAGE under reducing and non-reducing conditions as described above.

<u>Protein purification</u>

Six liters of conditioned medium from SK-LMS-1 cells grown in 175-cm^2 T flasks were prefiltered through a 0.5-μm filter (Millipore HAWP 142 50), and concentrated to 300ml by a Pellicon cassette system having a 10 kD molecular mass cutoff (Millipore PTGC 000 05). Concentrated medium was loaded onto heparin-Sepharose resin (4 ml. bed volume, LKB/Pharmacia) that had been equilibrated in 20mM Tris-HCl, pH7.5/0.3 M NaCl. The sample was eluted with a modified linear gradient of increasing NaCl concentration. Aliquots from each fraction were subjected to immunoblot analysis with antiserum raised against HGF (final dilution 1:500) to identify the presence of HGF/NK2. Pooled fractions were further resolved on a TSK G3000 sizing column (LKB/Pharmacia) in 20mM Tris-HCl, pH6.8/1.0 M NaCl. The purity and identity of the HGF/NK2 protein were determined by silver-stain analysis (Merril et al. *Science* 211:1437-1438 (1981)) and immunoblotting under reducing and non-reducing conditions. Fractions containing >95% of HGF/NK2 were selected for biological analysis. Protein concentration was estimated by optical density, assuming $A^{1\%}_{214}$ = 140.

Affinity cross-linking

TSK-purified HGF/NK2 was iodinated by the chloramine-T method (W.M. Hunter and F.C. Greenwood *Nature* 194:495-496 (1962)) and represented over 99% of the labeled material in the preparation as determined by SDS-PAGE analysis. Affinity cross-linking experiments were performed on 6-well plates seeded with B5/589 cells at a density of $5x10^5$ per well. To each well, HGF/NK2 ($5x10^5$ cpm at a specific activity of ~200 μCi/μg was added with or without cold competitors in HEPES binding buffer (100mM HEPES, 150mM NaCl, 5mM KCl, 1.2mM $MgSO_4$, 8.8mM dextrose, 2μg/ml heparin, and 0.1% BSA, pH7.4). Following incubation at room temperature for 45 minutes, cells were washed twice in cold HEPES saline (pH 7.4). Disuccinimidyl suberate (Pierce) in dimethyl sulfoxide was added to a final concentration of 250 μM and incubated for 15 min. Samples were then quenched with 100μl of 20mM Tris /100mM glycine /1mM EDTA for 1 minute and rinsed in HEPES saline. Cells were extracted with Laemmli sample buffer and resolved on 6.5% SDS-PAGE under reducing conditions.

Example 1. <u>Detection of a small naturally occurring HGF immunoreactive species and its putative transcrupt</u>

Previous studies demonstrated that HGF is synthesized as a single-chain poly-peptide with an apparent molecular mass (Mr) of 87,000 (87 kD). It can be cleaved into a heterodimeric form consisting of a heavy- (M_r 60 kD) and light-chain (M_r ~30 kD) held together by disulfide bonds. Neutralizing antiserum against purified HGF was used to immunoprecipitate proteins in conditioned medium from metabollically labeled M426 human embyonic lung fibroblasts. When sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions, the single-chain form (HGFp87) was the predominant species. While there was no evidence of the processed heavy- and light- chains, low levels of a HGF immunoreactive molecule of M_r ~34 kD (p34) were observed (Figure 1). Pulse chase experiments showed that both HGFp87 and p34 shared similar kinetics of synthesis and secretion arguing against the liklihood that p34 was a HGFp87 degradation product. When the same experiment was performed with another HGF-producer, a leiomyosarcoma cell line (SK-LMS-1), a similar pattern was seen except that p34 was relatively more abundant (Figure 1).

To gain further understanding of the relationship between HGFp87 and p34, poly(A)$^+$ RNA was prepared from M426 and SK-LMS-1 cells and subjected to Northern blot analysis using the full-length HGF coding sequence as probe. As shown in Figure 2, two major transcripts of 6.0 and 3.0

kilobases (kb) were detected in both lines. Each of these transcrupts has previously been shown to encode the full-length growth factor (Rubin et al., *Proc. Natl. Acad. Sci. USA* 88:415 (1991)). A third HGF hybridizing RNA of ~ 1.3 kb was present at a relatively low level in M426 cells, but was expressed at higher levels in SK-LMS-I cells. This pattern was consistant with the relative levels of p34 observed in the two cell lines, suggesting that p34 might be encoded by the novel 1.3 kb transcript. Based on the fact that the complete HGF coding sequence is ~ 2.0 kb, the 1.3 kb transcript could only repre: ıt a protion of this region. To t-st this, the same Nothern blot was hybridyzed separately with probes derived from either the N-terminal heavy-chain or the C-terminal light-chain. Whereas both probes were able to detect the 6.0 and 3.0 kb transcripts, only the heavy-chain probe was capable of recognizing the 1.3 kb message (Figure 2). These results suggested that this RNA species encoded a truncated version of the HGF molecule containing sequences from its N-terminal region.

Other faint bands were also detected in the Northern blots hybridized with probes derived from HGF (Figure 2), including one at approximately 2.2 kb. The significance of this observation became apparent after further study (see Example 2).

Example 2. <u>Isolation of HGF cDNA clones encoding only the N-terminal and first one or two kringle domains</u>

In an attempt to isolate cDNA clones corresponding to the 1.3 kb transcript, an M426 cDNA

library was differential scr ened with both HGF heavy- and light-chain probes. Clones that specifically hybridized to the heavy- but not the light-chain probe were plaque purified. Based on the sizes and physical maps of the inserts, one cDNA clone, pH45 with an insert of ~1.2 kb was selected for sequencing. As shown schematically in Figure 4A, clone pH45 depicted a transcript of 1199 basepairs (bp) composed of a short 5'-untranslated region of 75 bp, an open reading frame of 870 bp and a 254 bp 3'-untranslated region containing a polyadenylation signal, AATAAA. The open reading frame predicted a 290 amino acid truncated version of HGF consisting of a signal peptide, an N-terminal domain (N), and the first two kringle domains (K1 and K2) with a calculated Mr of ~30kD excluding the signal peptide. This sequence, which is designated NK2 was identical to that of HGF cDNA until it diverged at a point which coincided precisely with the end of the K2 domain. The NK2 open reading frame continued for two additional amino acids followed by an in-frame stop codon (TAA) (Figure 3 and 4A).

To ascertain the authenticity of the cDNA clone, polymerase chain reaction (PCR) analysis was performed with primers P1 and P2 (Figure 4A), the latter of which was specific for the NK2 transcript. Figure 4B shows the existence of the predicted 220 bp PCR fragment in RNA of M426 and SK-LMS-1 cells but not in B5/589 cells, which lack detectable HGF transcripts. The gene structure of this region was further analyzed by amplifying the corresponding genomic sequence using the same PCR primers (Figure

4B). Sequencing of the PCR product revealed a ~400 bp intron with the consensus splice donor/acceptor sequences CG/GT and AG/AG at the intron-exon boundaries, which aligned precisely with the predicted splice junction in the NK2 cDNA clone (Figure 4A). Thus, the 1.3 kb NK2 transcript is likely generated during precursor RNA processing by joining of the K2 exon to an alternative exon containing a termination codon instead of the K3 exon.

Using the differential screening strategy described above, three additional cDNA clones that specifically hybridized to the HGF heavy as opposed to the light chain probe were isolated from the M426 library. One of these was ~1.6 kb and contained the coding sequence for HGF/NK2; it differed from the 1.3 kb transcript only insofar as it included a longer stretch of 3' untranslated sequence. However, the other two inserts, one 1.5 and the other 2.2 kb, encoded only the N-terminal and first kringle domain; they differed from each other in their 3' untranslated regions. The coding sequence of one of these NK1 cDNAs is presented in Figure 9. As noted in the previous section, a close examination of the HGF hybridization pattern in Northern blot analysis revealed a weak 2.2 kb band as well as a diffuse signal at 1.3 -1.6 kb (Figure 2) which probably represents the transcripts corresponding to these low abundance cDNAs.

Example 3. Recombinant expression of HGF/NK2 cDNA identifies its product as the small HGF cross-reactive species

In order to test whether the NK2 transcript encodes the p34 protein detected in M426 and SK-LMS-1 cells, the NK2 coding region was subcloned into the expression vector, pCDV-1, in both anti-sense (pC45as) and sense (pC45s) orientations. Conditioned medium of COS-1 cells transfected with either construct was collected and immunoprecipitated with HGF neutralizing antibodies followed by SDS-PAGE analysis. As shown in Figure 5A, pC45s transfected COS-1 cells secreted a 34 kD HGF immunoreactive recombinant protein (rHGF/NK2) not detected when COS-1 cells were transfected with the pC45as construct. The size of this protein corresponded closely to that of p34 from M426 and SK-LMS-1 cells (Figure 5A). When the same experiment was performed under non-reducing conditions, the mobility of both recombinant and naturally occuring p34 shifted to an apparent Mr of ~28 kD (Figure 5B), providing further evidence that p34 and rNK2 were structurally indistinguishable.

The next experiment compared the heparin-binding properties of p34 and rHGF/NK2. Conditioned medium collected from SK-LMS-1 and pC45s-transfected COS-1 cells were each applied to heparin-Sepharose resin, and bound proteins were eluted with increasing NaCl concention. When individual fractions were analyzed by immunoblotting with anti-HGF serum, both p34 and rHGF/NK2 shared the same chromatographic profile with an elution peak at ~1.0M NaCl. Taken together, these findings

indicated that the p34 protein s creted by M426 and SK-LMS-1 cells represented a truncated version of HGF expressed from the NK2 transcript. Thus, the p34 protein was designated as HGF/NK2.

The NK2 coding region was also subcloned into the pZ1Pneo expression vector and subsequently transfected into NIH/3T3 mouse fibroblasts. The metabolically labeled protein was detected in the condition medium of transfected cells, but levels were not sufficient for preparative work.

Example 4. Purified HGF/NK2 is a specific inhibitor of HGF mitogenic activity

To investigate its biological activity, HGF/NK2 was purified from culture fluids of SK-LMS-1 cells by a three-step procedure combining ultrafiltration, heparin-Sepharose and TSK sieving chromatography. The purified protein exhibited the characteristic mobility shift under non-reducing and reducing conditions and was immunoreactive with anti-HGF serum, thereby confirming its identity as HGF/NK2 (Figure 6).

To test the mitogenic activity of HGF/NK2, a human mammary epithelial cell line, B5/589 was used as the target cell. While HGF stimulated [^{3}H]-thymidine incorporation with a half-maximal effect at ~0.25nM, under identical conditions HGF/NK2 at concentrations as high as 10nM caused no enhancement of DNA synthesis (Figure 7A). In view of their structural similarity, the possibility that HGF/NK2 might act as a specific inhibitor of HGF was also tested. When DNA synthesis induced by HGF was measured in the presence of increasing HGF/NK2

concentrations, a dose-dependent inhibition of [^{3}H]-thymidine incorporation was observed (Figure 7B). To achieve a 50% inhibition, a 10- to 20-fold molar excess of HGF/NK2 over HGF was required. Similar results were obtained when human melanocytes were used as target cells. Moveover, the inhibition was specific for HGF since HGF/NK2 did not impair the mitogentic activity of epidermal growth factor (EGF)(Figure 7B).

Example 5. Competitive binding of HGF/NK2 and HGF to the HGF receptor

It was recently demonstrated that the *c-met* protooncogene product, a membrane-spanning tyrosine kinase, is the cell surface receptor for HGF (Bottaro et al., *Science* 251:802 (1991)). To elucidate the mechanism by which HGF/NK2 acted as an antagonist of HGF mitogenic activity, cross-linking studies of [^{125}I]-HGF/NK2 to B5/589 cells were performed. As shown in Figure 8, a single major cross-linked species of 170 kD was detected under reducing conditions. This band corresponds to the 145 kD ß-subunit of the processed *c-met* product cross-linked to HGF/NK2 (Bottaro et al., *Science* 251:802 (1991)). Increasing concentrations of either unlabeled HGF/NK2 or HGF effectively competed with the labeled ligand in the cross-linking reaction. On a molar basis, HGF was estimated to be 3 to 5 times more effective than HGF/NK2 itself as a competitor of [^{125}I]-HGF/NK2 cross-linking. Under the same conditions, EGF failed to block HGF/NK2 cross-linking (Figure 8). All of these findings

demonstrate specific competitive binding of HGF/NK2 and HGF to the same cell surface receptor molecule.

* * * *

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

The entire contents of all references cited above are incorporated herein by reference.

<u>What is Claimed is</u>:

1. A hepatocyte growth factor (HGF) variant protein having an apparent molecular weight of about 34 kd as determined by SDS-PAGE under reducing conditions which specifically inhibits HGF-induced mitogenesis by binding to the HGF receptor.

2. A DNA segment encoding said HGF variant protein according to Claim 1.

3. The DNA segment according to claim 1 wherein said variant has the amino acid sequence defined in Figure 3.

4. A DNA segment encoding a hepatocyte growth factor variant, wherein said variant has the nucleotide sequence defined in Figure 9 or allelic sequence variations thereof.

5. A DNA fragment having the nucleotide sequence as defined in Figure 3 or allelic sequences variations thereof.

6. A recombinant DNA molecule comprising a DNA segment according to claim 2 and a vector.

7. A recombinantly produced protein having an amino acid sequence given in Figure 3.

8. A host cell stably or transiently transfected with the DNA segment according to claim 2 in a

manner allowing expression of said protein encoded in said DNA fragment.

9. A method of producing a recombinant 34 kd HGF variant protein comprising culturing host cells according to claim 10 or 11 in a manner allowing expression of said protein and isolating said protein from said host cells.

10. A method of producing a HGF variant (p 34) from cultured cells comprising the following steps:

(i) culturing HGF variant-producing cells in culture medium under conditions such that HGF variant is produced;

(ii) concentrating said culture medium so that a concentrate is formed;

(iii) contacting said concentrate with heparin under conditions such that HGF variant in said concentrate binds to the heparin whereby a heparin-HGF variant complex is formed;

(iv) separating said heparin-HGF variant complex from said concentrate;

(v) treating said heparin-HGF variant complex under conditions such that said HGF variant dissociates from the heparin so that a solution of free HGF variant is formed;

(vi) fractionating said solution by sizing chromatography so that HGF variant is separated from the remaining components.

11. A method of producing HGF variant from cultured cells according to claim 10 wherein said HGF variant-producing cells are human leiomyosarcoma SK-LMS-1 cells.

12. A method of inhibiting the growth of cells comprising contacting said cells with a therapeutic amount of the HGF variant according to claim 1 under conditions such that cell growth is inhibited.

13. A method of diagnosing growth disorders comprising the steps of:
(i) isolating mRNA from a biological sample
(ii) contacting said mRNA with a DNA segment according to claim 2 and
(iii) detecting the present of RNA-DNA hybrids.

14. A method of diagnosing growth disorders by *in situ* hybridization comprising the steps of:
(i) contacting mRNA contained in a biological sample with a DNA segment according to claim 2 or a RNA segment derived therefrom and
(ii) detecting the presence of RNA-DNA or RNA-RNA hybrids.

FIGURE 1

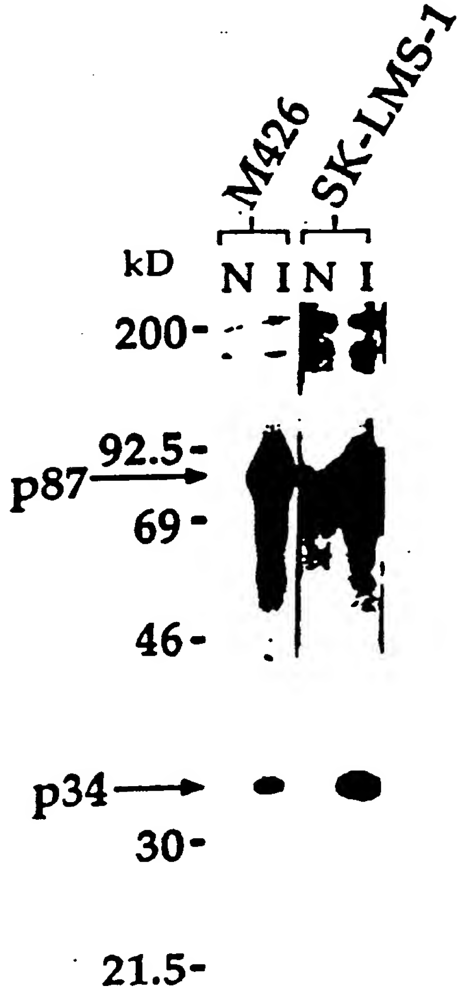


 FIGURE 2

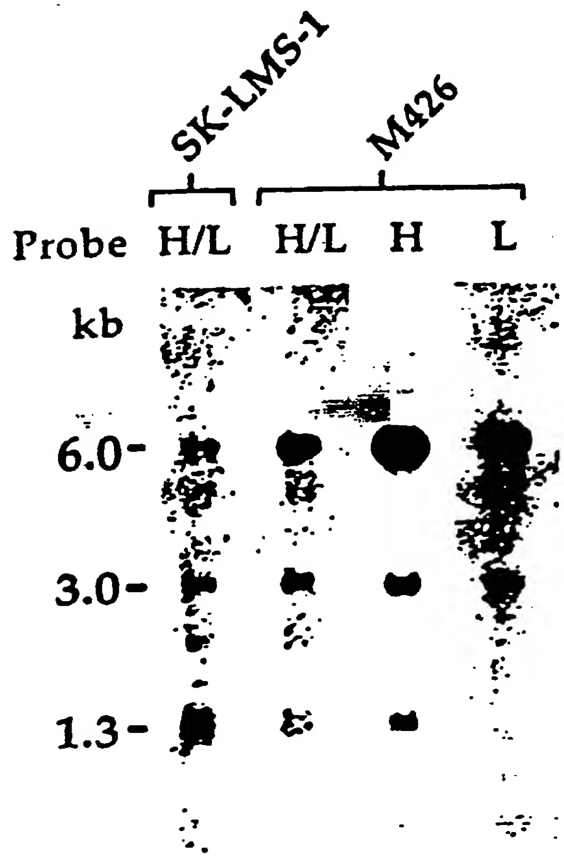


FIGURE 3

NK2 Coding Sequence

27 54
ATG TGG GTG ACC AAA CTC CTG CCA GCC CTG CTG CTG CAG CAT GTC CTC CTG CAT
MET Trp Val Thr Lys Leu Leu Pro Ala Leu Leu Leu Gln His Val Leu Leu His

81 108
CTC CTC CTG CTC CCC ATC GCC ATC CCC TAT GCA GAG GGA CAA AGG AAA AGA AGA
Leu Leu Leu Leu Pro Ile Ala Ile Pro Tyr Ala Glu Gly Gln Arg Lys Arg Arg

135 162
AAT ACA ATT CAT GAA TTC AAA AAA TCA GCA AAG ACT ACC CTA ATC AAA ATA GAT
Asn Thr Ile His Glu Phe Lys Lys Ser Ala Lys Thr Thr Leu Ile Lys Ile Asp

189 216
CCA GCA CTG AAG ATA AAA ACC AAA AAA GTG AAT ACT GCA GAC CAA TGT GCT AAT
Pro Ala Leu Lys Ile Lys Thr Lys Lys Val Asn Thr Ala Asp Gln Cys Ala Asn

243 270
AGA TGT ACT AGG AAT AAA GGA CTT CCA TTC ACT TGC AAG GCT TTT GTT TTT GAT
Arg Cys Thr Arg Asn Lys Gly Leu Pro Phe Thr Cys Lys Ala Phe Val Phe Asp

297 324
AAA GCA AGA AAA CAA TGC CTC TGG TTC CCC TTC AAT AGC ATG TCA AGT GGA GTG
Lys Ala Arg Lys Gln Cys Leu Trp Phe Pro Phe Asn Ser MET Ser Ser Gly Val

351 378
AAA AAA GAA TTT GGC CAT GAA TTT GAC CTC TAT GAA AAC AAA GAC TAC ATT AGA
Lys Lys Glu Phe Gly His Glu Phe Asp Leu Tyr Glu Asn Lys Asp Tyr Ile Arg

405 432
AAC TGC ATC ATT GGT AAA GGA CGC AGC TAC AAG GGA ACA GTA TCT ATC ACT AAG
Asn Cys Ile Ile Gly Lys Gly Arg Ser Tyr Lys Gly Thr Val Ser Ile Thr Lys

459 486
AGT GGC ATC AAA TGT CAG CCC TGG AGT TCC ATG ATA CCA CAC GAA CAC AGC TTT
Ser Gly Ile Lys Cys Gln Pro Trp Ser Ser MET Ile Pro His Glu His Ser Phe

513 540
TTG CCT TCG AGC TAT CGG GGT AAA GAC CTA CAG GAA AAC TAC TGT CGA AAT CCT
Leu Pro Ser Ser Tyr Arg Gly Lys Asp Leu Gln Glu Asn Tyr Cys Arg Asn Pro

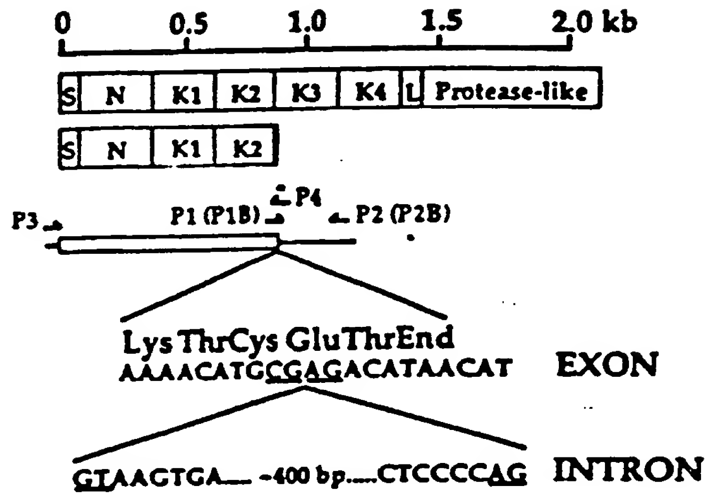
567 594
CGA GGG GAA GAA GGG GGA CCC TGG TGT TTC ACA AGC AAT CCA GAG GTA CGC TAC
Arg Gly Glu Glu Gly Gly Pro Trp Cys Phe Thr Ser Asn Pro Glu Val Arg Tyr

FIGURE 3 (CONT'D)

```
                                621                                             648
GAA GTC TGT GAC ATT CCT CAG TGT TCA GAA GTT GAA TGC ATG ACC TGC AAT GGG
Glu Val Cys Asp Ile Pro Gln Cys Ser Glu Val Glu Cys MET Thr Cys Asn Gly

                                675                                             702
GAG AGT TAT CGA GGT CTC ATG GAT CAT ACA GAA TCA GGC AAG ATT TGT CAG CGC
Glu Ser Tyr Arg Gly Leu MET Asp His Thr Glu Ser Gly Lys Ile Cys Gln Arg

                                729                                             756
TGG GAT CAT CAG ACA CCA CAC CGG CAC AAA TTC TTG CCT GAA AGA TAT CCC GAC
Trp Asp His Gln Thr Pro His Arg His Lys Phe Leu Pro Glu Arg Tyr Pro Asp

                                783                                             810
AAG GGC TTT GAT GAT AAT TAT TGC CGC AAT CCC GAT GGC CAG CCG AGG CCA TGG
Lys Gly Phe Asp Asp Asn Tyr Cys Arg Asn Pro Asp Gly Gln Pro Arg Pro Trp

                                837                                             864
TGC TAT ACT CTT GAC CCT CAC ACC CGC TGG GAG TAC TGT GCA ATT AAA ACA TGC
Cys Tyr Thr Leu Asp Pro His Thr Arg Trp Glu Tyr Cys Ala Ile Lys Thr Cys

GAG ACA TAA
Glu Thr  .
```

FIGURE 4

A


0
0.5
1.0
1.5
2.0 kb
S
N
K1
K2
K3
K4
L
Protease-like
S
N
K1
K2
P3
P1 (P1B)
P4
P2 (P2B)
Lys Thr Cys Glu Thr End
AAAACATGCCGAGACATAACAT
EXON
GTAAGTGA.... ~400 bp.....CTCCCCAG
INTRON

B

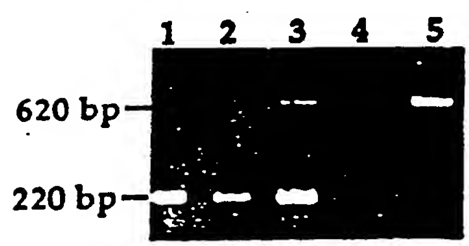

1
2
3
4
5
620 bp
220 bp

FIGURE 5

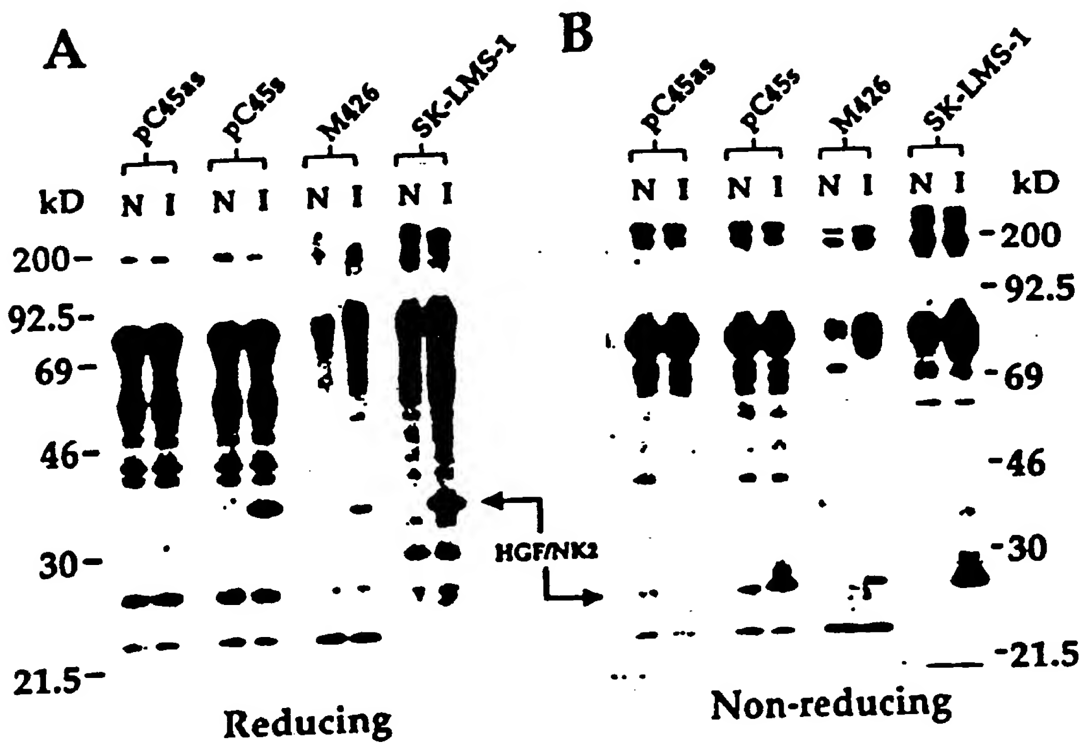

A
B
pC45as
pC45s
M426
SK-LMS-1
N I
kD
200-
92.5-
69-
46-
30-
21.5-
HGF/NK2
Reducing
Non-reducing

FIGURE 6

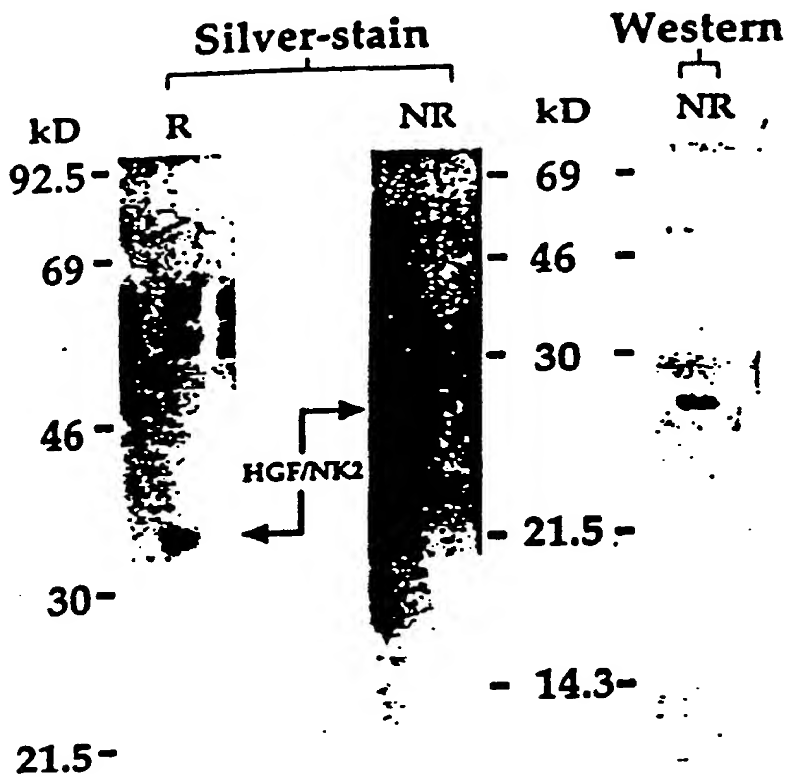

Silver-stain
Western
kD
R
NR
kD
NR
92.5
69
46
30
21.5
69
46
30
21.5
14.3
HGF/NK2

FIGURE 7

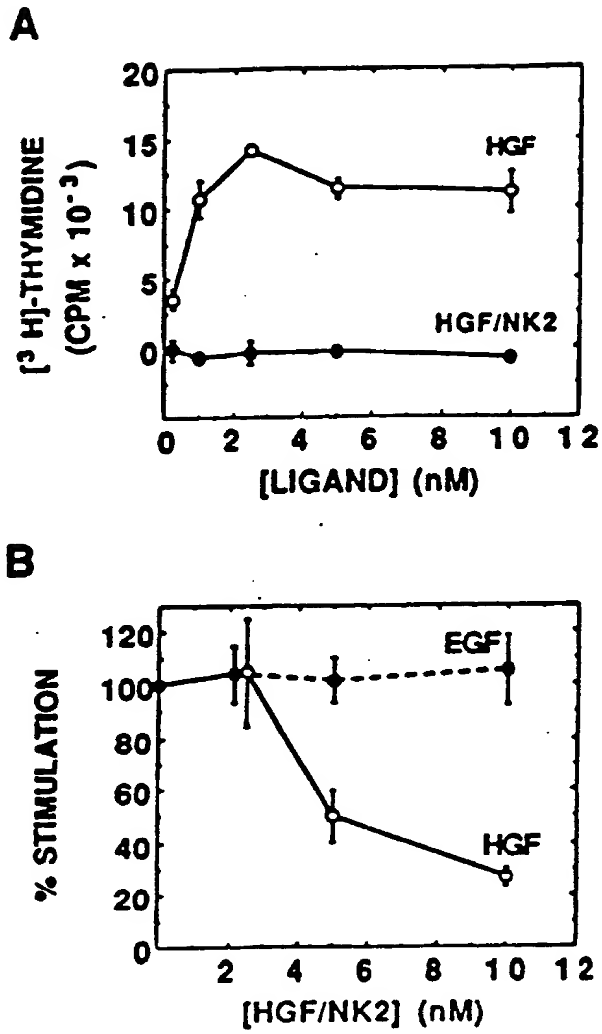

A
20
15
10
5
0
[3 H]-THYMIDINE
(CPM x 10^-3)
HGF
HGF/NK2
0 2 4 6 8 10 12
[LIGAND] (nM)
B
120
100
80
60
40
20
0
% STIMULATION
EGF
HGF
2 4 6 8 10 12
[HGF/NK2] (nM)

FIGURE 8

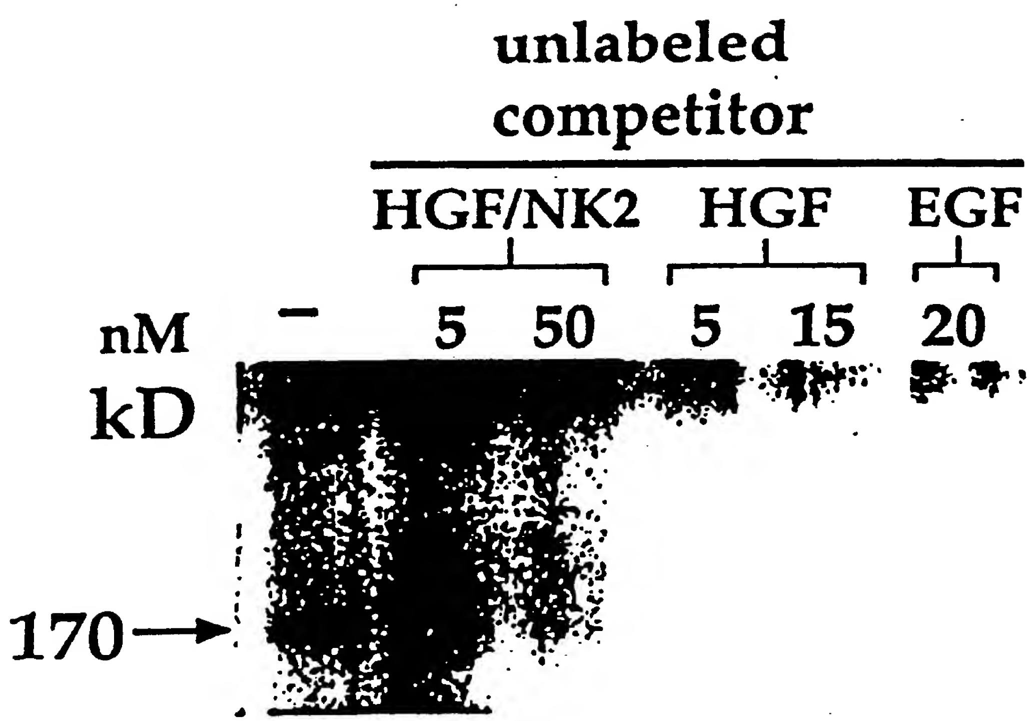


FIGURE 9

NK1 Coding Sequence

```
                                                27                                     54
ATG TGG GTG ACC AAA CTC CTG CCA GCC CTG CTG CTG CAG CAT GTC CTC CTG CAT
MET Trp Val Thr Lys Leu Leu Pro Ala Leu Leu Leu Gln His Val Leu Leu His

                                                81                                    108
CTC CTC CTG CTC CCC ATC GCC ATC CCC TAT GCA GAG GGA CAA AGG AAA AGA AGA
Leu Leu Leu Leu Pro Ile Ala Ile Pro Tyr Ala Glu Gly Gln Arg Lys Arg Arg

                                               135                                    162
AAT ACA ATT CAT GAA TTC AAA AAA TCA GCA AAG ACT ACC CTA ATC AAA ATA GAT
Asn Thr Ile His Glu Phe Lys Lys Ser Ala Lys Thr Thr Leu Ile Lys Ile Asp

                                               189                                    216
CCA GCA CTG AAG ATA AAA ACC AAA AAA GTG AAT ACT GCA GAC CAA TGT GCT AAT
Pro Ala Leu Lys Ile Lys Thr Lys Lys Val Asn Thr Ala Asp Gln Cys Ala Asn

                                               243                                    270
AGA TGT ACT AGG AAT AAA GGA CTT CCA TTC ACT TGC AAG GCT TTT GTT TTT GAT
Arg Cys Thr Arg Asn Lys Gly Leu Pro Phe Thr Cys Lys Ala Phe Val Phe Asp

                                               297                                    324
AAA GCA AGA AAA CAA TGC CTC TGG TTC CCC TTC AAT AGC ATG TCA AGT GGA GTG
Lys Ala Arg Lys Gln Cys Leu Trp Phe Pro Phe Asn Ser MET Ser Ser Gly Val

                                               351                                    378
AAA AAA GAA TTT GGC CAT GAA TTT GAC CTC TAT GAA AAC AAA GAC TAC ATT AGA
Lys Lys Glu Phe Gly His Glu Phe Asp Leu Tyr Glu Asn Lys Asp Tyr Ile Arg

                                               405                                    432
AAC TGC ATC ATT GGT AAA GGA CGC AGC TAC AAG GGA ACA GTA TCT ATC ACT AAG
Asn Cys Ile Ile Gly Lys Gly Arg Ser Tyr Lys Gly Thr Val Ser Ile Thr Lys

                                               459                                    486
AGT GGC ATC AAA TGT CAG CCC TGG AGT TCC ATG ATA CCA CAC GAA CAC AGC TTT
Ser Gly Ile Lys Cys Gln Pro Trp Ser Ser MET Ile Pro His Glu His Ser Phe

                                               513                                    540
TTG CCT TCG AGC TAT CGG GGT AAA GAC CTA CAG GAA AAC TAC TGT CGA AAT CCT
Leu Pro Ser Ser Tyr Arg Gly Lys Asp Leu Gln Glu Asn Tyr Cys Arg Asn Pro

                                               567                                    594
CGA GGG GAA GAA GGG GGA CCC TGG TGT TTC ACA AGC AAT CCA GAG GTA CGC TAC
Arg Gly Glu Glu Gly Gly Pr  Trp Cys Phe Thr S r Asn Pr  Glu Val Arg Tyr
```

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06368

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): C07H 15/12,17/00; C07K 3/00,13/00; C12N 1/20,15/00; A61K 37/24,37/36; C12P 19/36; C12Q 1/68; G01N 33/34 US Cl.:435/ 6,91,172.3,252.3; 436/163,501,811; 530/399

II. FIELDS SEARCHED ~~US Cl.: 435/6,91,172.3,252.3; 436/ 163,501,811; 530/399~~

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US	435/6,91,172.3,252.3; 436/163,501,811; 530/399

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,Y	WO,A, 90/10651 (Higashio et al.) 20 September 1990, see abstract and Figures 15 and 16.	1-14

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search 11 December 1991	Date of Mailing of this International Search Report 07 JAN 1992
International Searching Authority ISA/US	Signature of Authorized Officer Lori Yuan

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ______, because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ......, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers ______, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attachment (Telephone practice)

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.